

Insect immune response to bacterial infection is mediated by eicosanoids

(cyclooxygenase/lipoxygenase/phospholipase A₂/Manduca sexta/Serratia marcescens)

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ABSTRACT Inhibition of eicosanoid formation in larvae of the tobacco hornworm *Manduca sexta*, using specific inhibitors of phospholipase A₂, cyclooxygenase, and lipoxygenase, severely weakened the ability of larvae to clear the bacterium *Serratia marcescens* from their hemolymph. The reduced capability to remove bacteria is associated with increased mortality due to these bacteria. There is a dose-dependent relationship between the phospholipase A₂ inhibitor dexamethasone and both the reduced bacterial clearance and increased larval mortality. The dexamethasone effects on larval survival were reversed by treatment with arachidonic acid. Maleic acid, a nonspecific antioxidant, did not interfere with the insects' ability to remove bacterial cells from hemolymph. The larvae were shown to contain all of the C₂₀ polyunsaturated fatty acids necessary for eicosanoid biosynthesis and to be capable of converting radioactive arachidonic acid into several primary prostaglandins. These results strongly suggest that eicosanoids mediate transduction of bacterial infection signals into the complex of cellular and humoral responses that comprise invertebrate immunity.

Invertebrate immunity is an amalgam of several distinct systems, both cellular and humoral, that operate in a more or less coordinated way to provide protection from invading pathogens (1). Cellular responses include phagocytosis, encapsulation, and various cytotoxic reactions such as release of lysozyme (1, 2) and the prophenoloxidase activating system (3). These hemocyte-mediated responses are complemented by antibacterial activity, such as lectins, which occur constitutively in invertebrate hemolymph (4). In addition to normally occurring factors, several antibacterial peptides are induced by bacterial infections [namely, the cecropins (4 kDa), attacins (21 to 23 kDa), dipterocins (8 kDa), and insect defensins (4 kDa)] (3, 5).

An important area of invertebrate immunity that has not been critically addressed concerns how the early responses to infection are mediated—that is, what is the sequence of biochemical events operative between microbial invasion and the appearance of defense mechanisms? To date, only two early steps in the immune response have been investigated. In one, immune-specific RNA was shown to be synthesized 2–4 hr after bacterial infection of the moths *Hyalophora cecropia* and *Galleria mellonella* (3). In the other, inducible lectins were synthesized in the flesh fly *Sarcophaga peregrina* almost immediately after their immune system was challenged (6). However, these gene-specific events follow from earlier steps in transduction of bacterial invasion signals into physiological defense responses.

Since many aspects of immunological defense in mammals are mediated by eicosanoid metabolites of arachidonic acid (7), we hypothesized that biosynthesis of eicosanoids is also an essential early step in mediation of the immune response in invertebrates. We tested this hypothesis by using an insect, the tobacco hornworm *Manduca sexta*. We report here that selective pharmaceutical inhibitors of eicosanoid biosynthesis compromise the hornworm's immune response to bacterial infection. Furthermore, hornworms so compromised died earlier in the course of infection than did control animals. These findings indicate that eicosanoid biosynthesis is a crucial early step in invertebrate immunity.

MATERIALS AND METHODS

Organisms. Larval tobacco hornworms were reared under standard culture conditions (8), at 28°C under a 16-hr light/8-hr dark photoperiod. Experiments used early fifth (last prepupal) instar prewandering larvae. The bacterium *Serratia marcescens* used in this study is a red-pigmented strain obtained from the culture collection, School of Biological Sciences at the University of Nebraska-Lincoln. Bacteria were grown in 50 ml of standard methods broth (Difco) on a waterbath shaker at 30°C and 120 rpm. The cells were used in midlogarithmic or stationary phase at a titer of 2.5–7.0 × 10⁷ colony-forming units (cfu) per ml.

Bacterial Recovery: Influence of Inhibitors. Test larvae were injected with either the phospholipase A₂ (PLA₂) inhibitor dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], the cyclooxygenase inhibitor indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid], the 5- and 12-lipoxygenase inhibitor esculetin (6,7-dihydroxycoumarin), the nonspecific antioxidant maleic acid (*cis*-butenedioic acid; Sigma), or a 1:1 combination of indomethacin and esculetin (all others from BioMol, Plymouth Meeting, PA). All compounds were intrahemocoelically injected into individual larvae at dosages of 50 μg per component in 5 μl of absolute ethanol. Injections were made by inserting the needle of a Hamilton 701 syringe dorsolaterally above the last two spiracles, moving the needle into the hemocoel parallel to the body wall to avoid injuring the alimentary canal, then depressing the plunger. Three to 10 min after injection of inhibitors, larvae were infected with *S. marcescens* by intrahemocoelic injection of ≈5 × 10⁵ bacterial cells in 10 μl of broth, on the opposite side in the manner described above. Control larvae were injected with 5 μl of absolute ethanol and then similarly infected with bacteria. After a 2.5-hr incubation at 30°C, 100 μl of hemolymph was withdrawn from each

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Abbreviations: PLA₂, phospholipase A₂; cfu, colony-forming units; PI, postinfection; PUFA, polyunsaturated fatty acid.

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insect by excising a proleg and collecting hemolymph in calibrated microcapillary pipettes. The hemolymph was placed in 10.0 ml of dilution broth [containing 1 g of tryptone and 2 g of sodium citrate (pH 7.2) per liter]. Duplicate 0.1-ml samples of 100- and 1000-fold hemolymph dilutions were plated on standard methods agar (Difco). The plates were allowed to dry, inverted, and then incubated at 30°C for 36–40 hr. Red bacterial colonies on each plate were then counted. In all bacterial recovery experiments, each agar plate represents a hemolymph sample withdrawn from individually identifiable larvae.

Control Experiments. Direct effects of the pharmaceutical inhibitors on bacteria were tested by adding the compounds to *S. marcescens* cultures; bacterial growth was unaffected in all cases. Similarly, injections of the inhibitors into *M. sexta* larvae unchallenged with bacteria did not affect subsequent development. We also considered the possibility that bacteria that may have leaked onto the outer surface of the larvae could have increased the apparent recovery of bacterial colonies after the 2.5-hr incubations. One hour after 10^5 bacterial cells were painted onto the outer surface of larvae, no living bacteria could be recovered, indicating that external bacteria did not mitigate recovery results. We also assayed 100 μ l of hemolymph from untreated larvae for the presence of *S. marcescens* by collecting the hemolymph under sterile conditions and plating it in duplicate directly onto standard methods agar. No bacterial colonies were recovered.

Dose-Response Relationship: Bacterial Recovery. Four groups of seven larvae each were treated with 0, 1.4×10^{-5} , 1.4×10^{-3} , or 1.4×10^{-1} μ g of dexamethasone in 5 μ l of ethanol and then infected with bacteria as described above. After a 1.0-hr incubation at 30°C, 100 μ l of hemolymph was withdrawn from each insect as described. The hemolymph was placed in 10.0 ml of dilution broth, and 0.1 ml was plated in duplicate on standard methods agar. The plates were incubated, and bacterial colonies were counted as described.

Dose-Response Relationship: Larval Survival. Four groups of 41 larvae each were injected with either 5 μ l of ethanol or with 1.4×10^{-5} , 1.4×10^{-3} , or 1.4×10^{-1} μ g of dexamethasone in 5 μ l of ethanol. Three to 10 min after injection of dexamethasone, larvae were infected with $\approx 5 \times 10^5$ cells of *S. marcescens* by intrahemocoelic injection. The larvae were held at 30°C, and the number of survivors in each group was counted at 12, 14, 16, 18, and 20 hr postinfection (PI).

Time Course of Bacterial Recovery. Two groups of 27 larvae each were treated with 5 μ l of ethanol or with 50 μ g of dexamethasone in 5 μ l ethanol and then infected with *S. marcescens* as described above. Small hemolymph samples (15 μ l) were serially withdrawn from individual larvae at 15, 30, 45, and 60 min PI by clipping the caudal horn. Hemolymph samples were plated, and red-pigmented bacterial colonies were counted as described.

Arachidonic Acid Rescue. Individuals in groups of 20–41 larvae each were injected with either 5 μ l of ethanol, 0.14 μ g of dexamethasone in 5 μ l of ethanol, or with 0.14 μ g of dexamethasone in 5 μ l of ethanol and 50.0 μ g of arachidonic acid (Sigma) and then infected as described above with bacteria. The larvae were held at 30°C, and the numbers of survivors in each group were counted at 2-hr intervals beginning 12 hr PI.

Statistical analyses on detectable *S. marcescens* colonies in the larval hemolymph were conducted by using the Wilcoxon two-sample rank test or the Kruskal-Wallis statistics, and survivorship data were analyzed by the $R \times C$ contingency test.

Determination of Polyunsaturated Fatty Acids (PUFAs). Total lipids were extracted from hemolymph and fat bodies from five fifth-instar larvae by the method of Bligh and Dyer (9). Phospholipids were isolated by thin-layer chromatography of total lipid extract (10). Purified phospholipids were

transmethylated by refluxing in acidified methanol for 1 hr at 85°C (11). The resulting fatty acid methyl esters were analyzed by gas chromatography and mass spectrometry (11).

Eicosanoid Biosynthesis. Fifth-instar *M. sexta* larvae were injected with 9.25×10^5 Bq of tritiated arachidonic acid ([5,6,8,9,11,12,14,15- 3 H]20:4; 2.22–3.70 TBq/mmol; New England Nuclear). After 1 min, 100 μ l of hemolymph was collected as described above. Eicosanoids were extracted three times with acidified ethyl acetate and then separated by thin-layer chromatography (12). Fractions were identified by cochromatography with authentic unlabeled standards, and radioactivity in each fraction was assayed by liquid scintillation counting on a LKB-Wallac 1219 Rackbeta counter at 66% counting efficiency.

RESULTS

Time Course of Bacterial Recovery. Fig. 1 shows that the median bacterial recovery from ethanol-treated control larvae was below the limits of detectability for the first hour PI ($<10^3$ cfu per ml of hemolymph; $n = 27$ for all time points), indicating that the larvae are able to remove injected bacteria from their hemolymph. In contrast, the dexamethasone-treated larvae yielded a median value of 1.8×10^4 cfu per ml of hemolymph at 15 min PI (different from controls, $H = 10.90$), which steadily increased to 2.3×10^4 , 3.3×10^4 , and 4.6×10^4 cfu per ml of hemolymph at 30 min ($H = 5.52$), 45 min ($H = 7.68$), and 60 min ($H = 11.94$) PI, respectively (Fig. 1; $n = 27$ for all points, except $n = 26$ at 60 min). Presumably this increase reflects bacterial growth in the hemolymph of immunocompromised larvae.

Bacterial Recovery: Influence of Inhibitors. The results of the bacterial recovery experiments are summarized in Fig. 2. Bacteria were recovered from significantly more larvae treated with eicosanoid biosynthesis inhibitors than from control larvae. Red-pigmented *S. marcescens* bacterial colonies were seen on 87% of the agar plates from dexamethasone-treated larvae, whereas only 21% of the plates from control larvae had visible colonies ($W = 361.5$, $P = 0.0007$). Fig. 2 shows that larvae treated with compounds that block only one of the two main PUFA oxygenation pathways

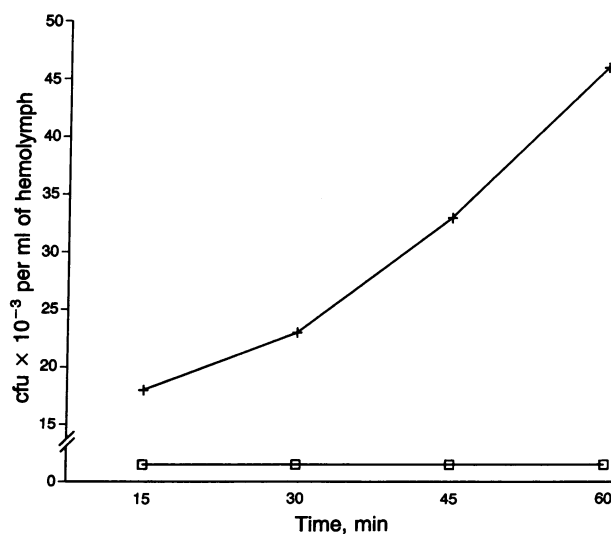


FIG. 1. Time course of recovery of the bacterial pathogen *S. marcescens* from larvae of *M. sexta*. Test larvae (+) were first injected with dexamethasone, and control larvae (\square) were first injected with ethanol. Both groups of larvae were then intrahemocoelically infected with $\approx 5 \times 10^5$ bacterial cells 3–10 min after treatment. At 15-min intervals PI, 15- μ l hemolymph samples were serially withdrawn from the caudal horn, diluted, and plated on standard methods agar plates.

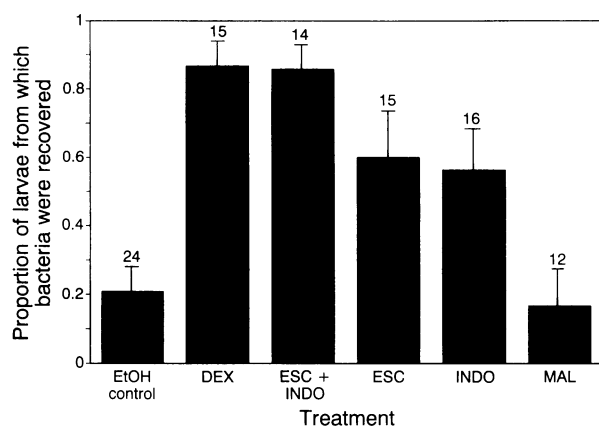


FIG. 2. Effect of treatment with eicosanoid biosynthesis inhibitors on immune response of *M. sexta* larvae to infection with the bacterial pathogen *S. marcescens*. Test larvae were first injected with dexamethasone (DEX), indomethacin (INDO), esculetin (ESC), maleic acid (MAL), or a 1:1 combination of esculetin and indomethacin (ESC + INDO). Control larvae were first injected with absolute ethanol (EtOH) only. Both test and control larvae were intrahemocoelically infected with $\approx 5 \times 10^5$ bacterial cells 3–10 min after treatment. After a 2.5-hr incubation, 100- μ l hemolymph samples were withdrawn, serially diluted, then plated on standard methods agar plates. The number of individual larvae per treatment is given above each treatment bar; the height of histogram bars represents the proportion of larvae from which bacteria were recovered, and the error bars represent 1 SE of the mean.

yielded intermediate results: colonies were recovered from about 58% of esculetin-treated larvae ($W = 409.5$, $P = 0.0433$) and from 55% of indomethacin-treated larvae ($W = 424.0$, $P = 0.0624$). When both major pathways were inhibited simultaneously with indomethacin and esculetin, bacterial colonies were recovered from 86% of the larvae, which is nearly identical to the dexamethasone effect ($W = 359.0$, $P = 0.0010$). Experiments with the nonspecific antioxidant compound maleic acid, which does not inhibit PUFA oxygenation pathways, yielded results identical to those of control larvae ($W = 450.0$, $P = 0.8536$).

Dose-Response for Dexamethasone: Bacterial Recovery. Dexamethasone treatments compromised the ability of hornworm larvae to clear *S. marcescens* from hemolymph samples taken at 1 hr after infection. Fig. 3 shows that increased dexamethasone dosages were associated with increased recovery of red-pigmented bacterial colonies. Hemolymph samples from larvae treated with ethanol or with 1.4×10^{-5} , 1.4×10^{-3} , or 1.4×10^{-1} μ g of dexamethasone per larvae yielded median values of 0.35×10^4 , 0.55×10^4 (not significant, $H = 0.722$), 8.2×10^4 (borderline significance, $H = 3.756$), and 10.1×10^4 (significant, $H = 5.409$) cfu per ml of hemolymph, respectively. Other experiments with higher dosages of dexamethasone (0.5 and 50.0 μ g per larvae) did not further increase recovery of bacterial colonies.

Dose-Response for Dexamethasone: Larval Survival. The strain of *S. marcescens* used in our experiments is pathogenic to *M. sexta* larvae: intrahemocoelic injection of ≥ 300 bacterial cells led to death within 24 hr. Since dexamethasone and the other inhibitors of eicosanoid biosynthesis compromised the ability of the larvae to clear bacteria from circulation, we expected similar treatments would also result in reduced survivorship during the first 24 hr PI. At 14 hr PI, 46.3% of the ethanol-treated control larvae were still alive. However (Fig. 4), only 9.8%, 24.4%, and 43.9% of larvae treated with 1.4×10^{-1} , 1.4×10^{-3} , or 1.4×10^{-5} μ g of dexamethasone per larvae survived 14 hr [$G(Yates)$ values compared to ethanol-treated controls were 12.50, 3.45, and 0.00, respectively].

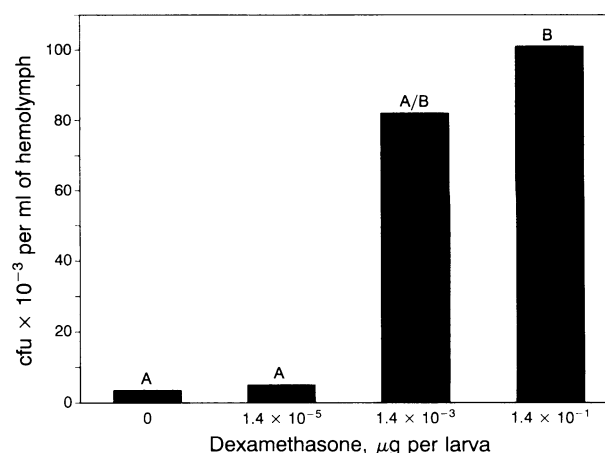


FIG. 3. Dose-response for dexamethasone: bacterial recovery. *M. sexta* larvae were first injected with 0.0–0.14 μ g of dexamethasone and then intrahemocoelically infected with 6×10^5 cells of the bacterium *S. marcescens*. After 1 hr at 30°C, hemolymph samples were withdrawn, diluted, and plated on standard methods agar plates. Red-pigmented bacterial colonies were counted 40–48 hr later. Columns with the same letters are not significantly different from each other (Kruskal-Wallis statistics).

The larval deaths could, of course, have been due to toxic effects of dexamethasone. We eliminated this possibility with a series of experiments by injecting unchallenged larvae with large doses (50 μ g) of dexamethasone, ethanol (the dexamethasone vehicle), the cell-free bacterial culture broth, and arachidonic acid. In all cases the larvae did not die during the 24-hr experimental period and showed no observable behavioral effects. These findings indicate that reduced ability to clear bacteria from hemolymph is associated with their earlier death from pathogenic infections.

Arachidonic Acid Reversed the Dexamethasone Effects. As we have just seen, dexamethasone greatly reduced the proportion of larvae that survived *S. marcescens* infections (Fig. 4). However, this dexamethasone effect was reversed by

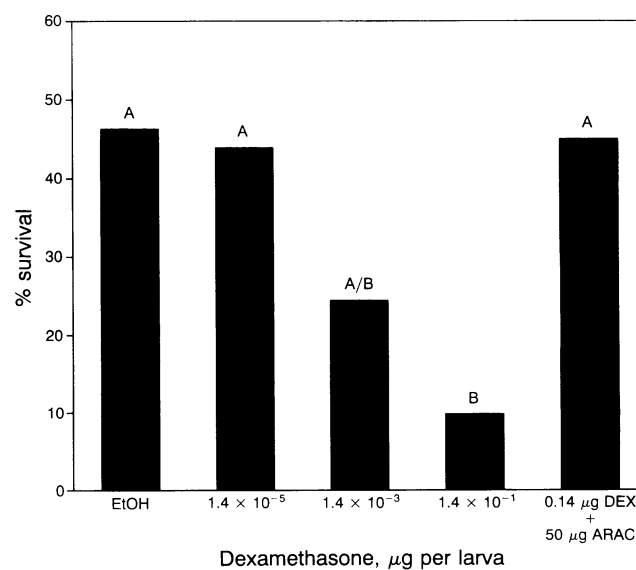


FIG. 4. Dose-response for dexamethasone (DEX): larval survival. *M. sexta* larvae were injected with 0.0–0.14 μ g of dexamethasone or with 0.14 μ g of dexamethasone plus 50 μ g of arachidonic acid (ARAC) and then intrahemocoelically infected with 6×10^5 cells of the bacterium *S. marcescens*. After 14 hr at 30°C, surviving larvae were counted. Bars with the same letter are not significantly different from each other ($R \times C$ contingency test).

treatment with arachidonic acid. At 14 hr PI, 45% of larvae treated with both dexamethasone and 50.0 μ g of arachidonic acid were alive, compared to 46.3% of ethanol-treated controls [$G(Yates) = 0.03$] and to 9.8% of larvae treated with 0.14 μ g of dexamethasone alone [$G(Yates) = 7.56$].

PUFA Composition. Analysis of fatty acid compositions of phospholipids purified from hemolymph showed that all three eicosanoid precursor PUFAs were present in *M. sexta*. These three components accounted for about 1% of total phospholipid fatty acids, comparable to results from many other terrestrial insect species (10); of these precursor PUFAs, 20:3 ($n = 6$) made up 20%, 20:4 ($n = 6$) made up 34%, and 20:5 ($n = 3$) made up 46%. Retention times and mass spectra of these compounds were exactly congruent with authentic standards. Thus, all potential eicosanoid precursors were present in this insect in sufficient quantities to support eicosanoid biosynthesis.

Eicosanoid Biosynthesis from Arachidonic Acid. Larvae of *M. sexta* were able to convert radioactive arachidonic acid into eicosanoids. One minute after injection, 100- μ l hemolymph samples were withdrawn from each of seven larvae. In these samples, 5.3% of the injected radioactivity (mean = 2.9×10^5 dpm) was recovered. Most (95–97%) of the recovered radioactivity was arachidonic acid. Three fractions (expressed as the percentage of recovered radioactivity ± 1 SD, $n = 7$) had chromatographic behavior identical to the prostaglandins F_{2a} ($0.1\% \pm 0.01\%$), E_2 ($0.1\% \pm 0.02\%$), and D_2 ($0.5\% \pm 0.2\%$). A similar amount of radioactivity was also recovered in an otherwise uncharacterized fraction in the same R_f range reported for lipoxigenase metabolites in mammalian systems, presumably hydroxyeicosatetraenoic acids (12).

DISCUSSION

In vertebrates and invertebrates, eicosanoids are formed from unesterified C_{20} PUFAs, which arise primarily by action of PLA2 on phospholipid components of membranes (13). Once available, these PUFAs can be oxygenated by two main routes: the cyclooxygenase pathway, which leads to the biologically active prostaglandins, and the lipoxigenase pathway, which is a complex of pathways leading to hydroperoxyeicosatetraenoic acids, hydroxyeicosatetraenoic acids, leukotrienes, lipoxins, and hepoxilins (13, 14). Products of both pathways are important in various aspects of mammalian host defense. For example, cyclooxygenase products have profound effects on macrophage locomotion, shape changes, and phagocytosis (15), whereas lipoxigenase products mediate chemotaxis, chemokinesis, and adherence responses of neutrophils (7).

The results presented in this paper strongly support our hypothesis that eicosanoids also mediate invertebrate immune responses. This view is summarized in the following arguments. First, inhibition of eicosanoid formation with specific inhibitors of PLA2, cyclooxygenase, and lipoxigenase significantly reduced the ability of larvae to clear pathogenic bacteria from their hemolymph. Second, the reduced capability to clear bacteria was associated with increased mortality caused by these bacteria. Third, for dexamethasone, both the increased bacterial recovery and decreased larval survival were expressed in a dose-dependent manner. Finally, the dexamethasone effects were reversed by treatment with arachidonic acid.

A major difficulty with using pharmaceutical inhibitors to probe biochemical pathways is the possibility of nonspecific or nonphysiological effects. The inhibitors used here are known to be very specific in mammals; less is known about their specificity in invertebrate systems. We feel the extrapolation to *M. sexta* is justified by four examples where these compounds have been shown to have similar pharmacolog-

ical action in invertebrates and mammals. First, acetylsalicylic acid (aspirin) inhibits prostaglandin biosynthesis in microsomal preparations from cockroach fat body (16), in *in vitro* preparations from house flies (17), and in reproductive tissues of the cricket *Teleogryllus commodus* (18). Second, indomethacin inhibits prostaglandin-mediated oviposition behavior in silk moths (19). Third, the PLA2 inhibitor dexamethasone mimics the effects of essential arachidonic acid deficiency in the mosquito *Culex pipiens* (20). Fourth, esculetin inhibits eicosanoid-mediated host penetration behavior of the blood fluke *Schistosoma mansoni* (21). Finally, the possibility of nonspecific effects was assessed by our use of high doses of maleic acid, a general antioxidant that does not interfere with cyclooxygenase or lipoxigenase enzymes. In these experiments, bacterial recoveries from larvae treated with maleic acid were exactly congruent with control larvae.

Dose-response relationships and end-product reversal provide crucial indications that inhibitors of eicosanoid biosynthesis interrupt a physiological link in invertebrate immune response to bacterial infections. Significantly fewer bacterial colonies were recovered from larvae that received no dexamethasone or very low doses of dexamethasone compared to larvae that were treated with higher doses. An analogous dose-response relationship was also observed in the survivorship experiments. Dexamethasone exerts its effects by inhibiting PLA2, which indirectly inhibits eicosanoid biosynthesis because substrate (i.e., unesterified arachidonic acid) is not available. If dexamethasone inhibited PLA2 in *M. sexta*, then treatment with arachidonic acid could be expected to ameliorate the effects on survivorship of infected larvae. Indeed, arachidonic acid treatment significantly reduced mortality to the level observed in ethanol-injected control larvae. These experiments support the idea that dexamethasone interrupts eicosanoid mediation of immune response in *M. sexta*.

The presence and biological significance of PUFAs and eicosanoids in insects are not yet widely appreciated (10, 13, 22). For this reason, when invoking eicosanoid mediation of an important physiological response in an invertebrate not yet studied in this regard, such as *M. sexta*, it is necessary to demonstrate that both substrate and eicosanoid biosynthetic activity are present. So far, there is only one other well-documented physiological role of eicosanoids in insects—namely release of egg-laying behavior in three species (23). Capillary column gas chromatography and mass spectrometric analysis confirmed the presence of all three known eicosanoid precursor PUFAs, 20:3 ($n = 6$), 20:4 ($n = 6$), and 20:5 ($n = 3$), in phospholipids from hemolymph of *M. sexta*. We also observed biosynthesis of eicosanoids from radioactive precursor in *M. sexta* hemolymph. This evidence shows that *M. sexta* is competent to biosynthesize eicosanoids from arachidonic acid. It is expected that a wide range of eicosanoid species will prove to be components of a complex regulatory system that initiates and terminates many elements of the immunological response in invertebrates, as seen in mammals (7).

Although we do not yet know the precise functions eicosanoids play in insect immunity, the timing of their action provides some insight. Our time course experiments show that the dexamethasone effect on clearing *S. marcescens* from hemolymph can be seen as early as 15 min PI. Rapid clearance of another insect pathogenic bacterium, *Pseudomonas aeruginosa* P11-1, from the hemolymph of *M. sexta* larvae has been described by Dunn and colleagues. This clearance was attributed to nodule formation at or near the injection site during the first 2 hr PI (24), followed by phagocytosis in the next 6 hr (25). They correlated these observations with hemocyte population dynamics during the first hour PI (26, 27). We suggest that eicosanoid metabolism mediates some/all of these early defense responses.

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